templates if distinguishable non-radioactive labels are present, are detected in or from a single lane of a sequencing gel or a single capillary electrophoresis tube or a single matrix or means of any kind using an automated sequencer or other detection device.

Examples

Example 1: Creation and Characterization of a Mutant T7 RNA Polymerase

A. Materials and Methods

Nucleic acids and NTPs: Nucleotides were from Pharmacia or USB/Amersham. Polynucleotides were from Pharmacia and the Midland Certified Reagent Company. Synthetic DNAs were prepared at the UTHSCSA DNA synthesis facility on an Applied Biosystems DNA synthesizer and purified by HPLC. A synthetic RNA 12mer was from the Midland Certified Reagent Company. Plasmids pT75 (Tabor and Richardson, 1985) and pBS (Stratagene Inc.) were purified from *E. coli* by alkaline-lysis and cesium chloride gradient centrifugation (Sambrook, et al., 1989). Radioactive nucleotides were from NEN Dupont or ICN.

Preparation and purification of mutant polymerases: Construction, expression, and purification of the T7 RNAP mutants was described previously (Bonner, et al., 1992).

Transcription reactions: Transcription reactions were carried out in 40 mM Tris-Cl pH 8.0, 15 mM $MgCl_2$, and 5 mM DTT or 20 mM Manganese Citrate pH 8.0, 5 mM DTT at 37°C. Template, polymerase, and NTP concentrations were as indicated in the legends to the figures and tables. Relative activity determinations were made by taking 4 µl aliquots of reactions at 5, 10, and 20 minute time points and spotting on to DE81 filter paper. Unincorporated

nucleotides were separated from incorporated nucleotides by washing the filter paper with .5 M KH_2PO_4 pH 7.0 and retained radioactive nucleotide was quantitated with a Molecular Dynamics phosphorimager. Radioactive NTPs used were as indicated in figure and table legends. To evaluate rNTP/dNTP selectivity, reactions were run with 4 rNTPs and pT75 as template and α-P^{32} rNTPs or α-P^{32} dNTPs were used to label the transcripts. The relative rate of incorporation of an rNTP vs. its cognate dNTP was determined from the relative percentages of labeled rNTP vs. dNTP incorporated into DE81 retainable RNA at 5, 10, and 20 minute time points. Apparent miscoding frequencies were determined similarly, though in this instance the template was a single-stranded homopolymer and the reaction contained the complementary unlabeled rNTP, and complementary α-P^{32} rNTP or one of the 3 non-complementary α-P^{32} rNTPs. The relative percentages of labeled complementary vs. non-complementary rNTPs incorporated at 5, 10 and 20 minute time points gave an apparent miscoding rate. The rNTP/dNTP selectivity assay was used to test the following T7 RNAP mutants for effects on substrate discrimination: D537S, D537E, S539A, R551S, D552S, R627S, K631S, L637A, Y639S, Y639F, Y639A, G640A, F644A, G645A, Q649S, I810S, H811S, H811A, D812A, D812E, D812S, D812N, D879E+ΔF882+ΔA883, D879E+A881T+ΔA883, D879E+F884+A885, D879E+F882Y, D879E+ΔA883, D879E+F882W, D879E.

Elongation rate determinations were carried out as described (Golomb & Chamberlin, 1974) with some variations (Bonner, et al., 1994).

Determination of NTP K_m and k_{cat} was as described previously (Patra, et al., 1992).

B. Results

Structure of the transcripts synthesized by Y639F and the w.t. enzyme with rNTPs and dNTPS: Fig. 1 diagrams the structure of transcription products produced by Y639F and w.t. T7 RNAP in the presence of various combinations of rNTPs and dNTPs. The template was pT75 (Tabor and Richardson, 1985) cut with *Hind*III so that transcription from its T7 promoter generates a 59-base run-off transcript. Electrophoresis was on a 20% polyacrylamide 6M urea gel. Plasmid and polymerases were at concentrations of 10^{-7} M, and NTP concentrations were .5 mM (all rNTPs and dTTP), 1 mM (dATP, dGTP), or 5 mM (dCTP). γ-P^{32}-GTP was added to radiolabel the transcription products. Wild-type (WT) or Y639F mutant (Mu) polymerases and NTPs used are as indicated. Poly-rG products of various sizes are labeled in lane A ("2G", "3G", etc...) and heterogeneous sequence abortive transcripts of different lengths are indicated by "4H", "5H", etc... in lane c. Lanes q-t are a 10-fold longer exposure of lanes m-p.

Fig. 1 shows transcription reactions carried out with the w.t. enzyme or the Y639F mutant polymerase and a T7 ϕ-10 promoter template. Transcription by T7 RNA polymerase, like other RNA polymerases, is characterized by an initial, poorly processive 'abortive' phase of transcription during which the short, nascent transcript frequently dissociates from the ternary complex. When the transcript reaches a length of ~9 bases transcription becomes highly processive and the transcript becomes stably associated with the elongation complex. On this promoter, which initiates with GGGAGACCGGAAU (SEQ ID NO:1), T7 RNAP can also synthesize long poly-G ladders (labeled "2G", 3G", etc. in lanes a and b) when rGTP is the sole NTP present (Martin, et al., 1988).